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PRINCIPAL INVESTIGATOR: Y. N. Vashisht Gopal, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030

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Y. N. Vashisht Gopal, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030
E-Mail: vynanda@mail.mdanderson.org

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13. ABSTRACT (Maximum 200 Words)

I had previously reported the identification of a hairpin polyamide that binds sequence specifically to the core promoter of the Her-2 oncogene and interferes with TBP binding. I had also constructed KRAB-fused transcriptional repressor proteins, which binds to the AP-2 binding site of the Her-2 promoter. I now report my findings on the transcriptional repression and proliferation inhibition carried out by the recombinant repressor proteins. The data presented in this report suggests that these recombinant repressors inhibit the expression of proteins involved in cancer cell survival and proliferation. The overall effect of these proteins results in apoptosis of Her-2 over-expressing cells. In conclusion, these recombinant proteins have the potential to be used as gene therapeutics for breast cancers that show Her-2 over-expression.

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Table of Contents

Introduction:

The Her-2/neu gene encodes a 185 Kd transmembrane receptor tyrosine kinase, which has partial homology with other members of the epidermal growth factor receptor (EGFR) family. Her-2 monomers dimerize or oligomerize with themselves or other members of the EGFR family and phosphorylate many downstream molecules, which leads to activation of a variety of signal transduction pathways (1, 2, 3, 4). Some of the well-known pathways are the PI3K/AKT, MAPK, cAMP/PKA (2, 3). Each of these pathways contributes to cell proliferation and cell survival. Oncogenic transformation by Her-2 is caused by disruption of cell cycle regulators (p21, p27), increased p53 degradation, stimulation of Wnt and NF-kB signaling pathways (reviewed in 5). Her-2 is overexpressed in brain, head & Neck, pancreatic, colorectal, prostrate, ovarian and most significantly in breast cancers (1, 6, 7, 8, 9). Thirty percent of all breast cancers show overexpressed Her-2, which causes a highly intractable malignant cancer phenotype and almost always spells out poor prognosis and therapeutic outcome (10, 11). Recent advances in the development of specific Her-2 targeting drugs like the antibody Herceptin have raised hopes for a better prognosis of Her-2 up-regulated cancer phenotype (12). However, development of resistance to Herceptin and other drugs is cause for concern. There is a great need for developing newer and more effective therapeutics is that target Her-2 at a primordial level, for example- during transcription of Her-2. Such therapeutics, while showing greater specificity, may also not be susceptible to the development of resistance since a transcriptional repressor of Her-2 would effectively reduce the levels of Her-2 and shut down important survival signaling pathways.

Summary of work from previous annual reports:

In the first year of report, I had shown that the unique DNA binding molecules called hairpin polyamides possess extremely high specificity and affinity for targeted DNA sequences. I had characterized the DNA interactions of two of these molecules (HPA-1 and HPA-2) using a high throughput methodology called REPSA and verified them using high resolution DNase I footprinting assays (figures 2 and 3 in 2002 annual report). The findings from this work have been published in the journal *Biochemistry*, 2003, The hairpin polyamide HPA-1 shows high specificity and affinity of interaction with the sequence '5-AGTATA-3', which is also part of the Her-2/neu promoter TATA box sequence. I have shown through an electrophoretic mobility shift assay (EMSA) that HPA-1 binds strongly to the TATA box sequence of the Her-2 promoter

and competitively inhibits the binding of basal transcriptional machinery with the TATA box (figure 4A in 2002 annual report). But HPA-1 shows weak *in vivo* inhibition of Her-2 transcription in cultured breast cancer cells (figure 4B in 2002 annual report, figure 1A in 2003 annual report). Due to this limitation, extremely high concentrations of HPA-1 are required to show therapeutic levels of Her-2 transcriptional inhibition. In our effort to develop potent Her-2 transcriptional inhibitors, we had introduced recombinant proteins, which show potent transcriptional repression of the Her-2 promoter activity. These recombinant proteins contain a strong transcriptional repression domain called KRAB (kruppel associated box) fused to a full length or DNA binding domain of AP-2alpha protein which, when introduced into cells, bind to the AP-2 binding sites in the Her-2 promoter. The domain structure of these recombinant fusion constructs is shown in Figure 1.

In about 70 % of Her-2 over-expressing cancers, AP-2 is also over-expressed and is known to activate Her-2 transcription, leading to aggressive growth of tumors. In the 2003 annual report, I showed through luciferase-reporter assays and immuno-fluorescence assays that expression of KRAB-AP2 recombinant fusion proteins KAP2 and KAP2r from plasmid constructs antagonizes the activation of Her-2 by AP-2, leading to repression of Her-2 transcription in breast cancer cells (figures 1B, 1C, 1D and 2 in 2003 annual report). This transcriptional repression resulted in decreased proliferation of Her-2 over-expressing cancer cells, but not the Her-2 normal cancer cells or normal breast epithelial cells (figure 3A in 2003 annual report). The hairpin polyamide HPA-1 however did not induce a strong repression of Her-2 activity (figure 1A in 2003 annual report) nor did it show a strong inhibition of cancer cell proliferation (figure 3B in 2003 annual report). A TUNEL (terminal deoxynucleotidyl transferase) assay showed that the decreased cancer cell proliferation in presence of the KRAB-recombinants was a result of apoptosis in the Her-2 over-expressing cells (figure 5 in 2003 annual report).

In the current report, we present detailed analysis of the expression of KRAB-recombinant constructs in SkBr-3 cells and show that apart from Her-2, these constructs affect other programs of gene expression that are important for cell growth and proliferation, leading to an overall effect of reduced proliferation and finally resulting in apoptosis of the cells.

BODY

Specific aims for the year of report.

A. Determination of the effect of Her-2 downregulation on the proliferative and physiological response of Her-2 over-expressing cancer cells.

B. Responsiveness of chemo-resistant breast cancers to chemotherapy in presence of the KRAB recombinant proteins.

Studies and results:

Determination of the effect of Her-2 downregulation on the proliferative and physiological response of Her-2 over-expressing cancer cells.

We had earlier used the WST-1 assay system (modified MTT assay which has superior sensitivity for cell proliferation studies) to show the effect of the KRAB-recombinants and HPA-1 on cancer cell proliferation (figure 3A and 3B in 2003 annual report). We also showed that KAP2 and KAP2r, when used in combination with HPA-1 induce synergistic inhibition Her-2 promoter activity (figure 1C in 2003 annual report) and a strong inhibition of cancer cell proliferation (figure 3C in 2003 annual report).

However, the proliferation assays carried out until now could reveal only partial effects because, in these experiments, only a fraction of the plasmid-transfected cells express the KRAB-recombinant proteins (in transient transfections using Fugene reagent). But the proliferation assays reveal information on transfected as well as untransfected cells. Hence, in these assays, absolute values of proliferation inhibition in cells that express the KRAB-recombinant proteins could not be obtained. In order to see the effect of KAP2 and KAP2r expression in only the transfected cells, we carried out a FACS (fluorescence activated cell sorting) analysis. Her-2 over-expressing SkBr-3 and Her-2 normal MCF-7 breast cancer cells were transfected with the plasmids expressing AP2, KAP2 or KAP2r and incubated for a period of 48 h. After the incubation, the cells were dissociated from the surface using a cell dissociation buffer (Life Technologies) and fixed with formaldehyde. The fixed cells were incubated for two hours with a FITC-tagged anti-FLAG antibody which recognizes a FLAG epitope on the recombinant AP-2 and KRAB-AP2 proteins. The cells were washed in phosphate buffered saline three times and then treated with propidium iodide. A cell cycle profile based on propidium iodide staining was generated for the cells. Cell cycle profiles of the FITC positive and FITC negative cells were

separated and plotted. The plots of the FITC positive cells would specify the cell cycle profile of a homogeneous population of the transfected cells that express the recombinant proteins. Shown in Figure 2, the different phases of the cell cycle are indicated as M1 (G1 phase), M2 (S phase), M3 (G2/M phase) and M4 (sub-G1 or apoptotic phase). The AP-2 transfected cells show moderate or basal level of apoptosis as observed in untransfected SkBr-3 cells, while the KAP2 and KAP2r transfected cells show high levels of apoptosis, as indicated by an increase in the sub-G1 population of the cells. MCF-7 cells however, do not show appreciable levels apoptosis in the presence of neither AP-2 nor KAP2 and KAP2r (Figure 3).

To substantiate the apoptosis caused by the KRAB-recombinants, a caspase assay was carried out in SkBr-3 cells transfected by these recombinants. In this assay, the cells were transfected with the plasmids expressing the recombinants and split into three sets of triplicates for each transfection. These were plated in 96 well plates and incubated for 40 h. After the incubation, the first set of triplicates was left untreated, the second set was treated with the caspase inhibitor z-VAD-FMK and the third set was treated with the apoptosis inducing ligand, TRAIL. After 8h (total 48 h incubation), the cells were processed using the Apo-One Caspase 3/7 assay system (Promega). In this caspase detection system, the cells are lysed, treated with a rhodamine tagged caspase 3/7 substrate (z-DEVD-R110) and incubated to allow caspase 3/7 cleavage of the substrate, which releases the rhodamine tag. Fluorescence quantification of this released rhodamine using absorption-485nm and emission-530nm wavelengths provides a direct measure of effector caspase activity, which in turn gives an indirect estimation of apoptosis. The data plotted in Figure 4 shows that the control plasmid transfected cells show a basal level of caspase activity after 48 h. The pan-caspase inhibitor z-VAD-FMK inhibited this caspase activity. This caspase inhibitor serves as an additional control to monitor fluorescence increase as authentic caspase activity. The apoptosis inducing ligand, TRAIL, which also serves as a control for monitoring caspase activity, increased caspase activity in the control cells, indicating apoptosis. Another control, which was transfected with a plasmid expressing only the KRAB domain also shows a similar effect. The AP2 transfected cells showed a small increase in caspase activity, while z-VAD-FMK and TRAIL in these cells showed similar effects as in the control. However the KRAB-recombinant proteins showed a very strong increase in caspase activity, indicating increased apoptosis in their presence. z-VAD-FMK inhibited this caspase activity to a greater extent, while TRAIL showed a high activity in these cells, indicating an overall increase in

apoptosis in the cells. The lower levels of caspase activity in the KRAB-recombinant transfected cells compared to the TRAIL treated cells is because of the limitation of transfection efficiency. The recombinants transfect only a fraction of the cells (~20%), while TRAIL essentially enters all cells. The limitation of transfection efficiency is common to all gene based therapeutics. While this limitation would adversely affect therapeutic usefulness, a pragmatic analysis of anticancer efficacy vis-à-vis toxicity should be conducted to evaluate any gene therapeutic. However, with continual advances in gene delivery mechanisms and tumor targeting methods, the therapeutic usefulness of gene-based systems could not be over-emphasized.

I had earlier carried out a colony formation assay to understand the effect of KRAB-recombinant expression on the proliferation of SkBr-3 cells. The data revealed that while cells transfected with AP-2 could form equal number of colonies as compared to cells transfected with a control plasmid in a G418 antibiotic restrictive environment, KAP2 and KAP2r could form only a few colonies (figure 4 in 2003 annual report). Western analysis revealed that the surviving colonies in the KAP2 and KAP2r transfected plates do not express the recombinant protein, strongly suggesting that while AP-2 expression provides a growth advantage to SkBr-3 cells, KAP2 and KAP2r provide a growth disadvantage. The same experiment carried out using MCF-7 cells also shows a similar trend, but not as pronounced as in SkBr-3 cells (Figure 5).

Because the KAP2 and KAP2r constructs confer a growth disadvantage to the cancer cells, I could not generate any stable clones of SkBr-3 cells that constitutively express these recombinant proteins. But in order to perform experiments for understanding the effect of the KRAB-recombinant proteins on cell physiology, one must have a homogeneous population of cells expressing these proteins. To do this, I developed a novel, yet simple FACS based methodology that would rapidly sort out transfected cells as a homogeneous population. In this methodology, the plasmid expressing a recombinant construct is transiently co-transfected into cells along with a GFP (Green Fluorescent Protein) expression vector and the GFP positive cells are selected by FACS. Co-transfection of cells with a 2:1 mix of recombinant plasmid and GFP vector with Fugene reagent has consistently shown that >99% of the FACS selected cells express the recombinant proteins. Up to a million positive cells can be isolated from cultures having low transfection efficiencies. Biochemical analysis (northern blotting, RT-PCR, western blotting) of these cells after 24 h to 36 h of transfection has excellent advantages compared to stable transfections. These advantages are enumerated below-

1. Due to mostly extra-chromosomal expression, the plasmids do not induce genetic variations in the cells and low levels of GFP expression do not have any noticeable effect on cellular physiology.
2. A short period of high-level expression is preferable for invoking targeted activity rather than a long-term stable expression that could possibly induce generalized effects and alter the genetic and physiological properties of cells.
3. The levels of recombinant protein expression in the cells can be controlled by the amount of plasmid being transfected.
4. Current inducible expression systems show leaky expression in un-induced cells. Leaky expression of AP-2 from un-induced cells has been reported to possess quantifiable transcriptional effects.
5. Virally transduced cells show immunogenic responses, which could interfere with the study of recombinant proteins.

Whole cell lysates were made from these sorted cells and tested for the levels of Her-2 and various other proteins involved in cell cycle regulation and malignant transformation using western blotting. The data shows that cells transfected with KAP2 and KAP2r have lower levels of Her-2, VEGF and Cyclin D1 and higher levels of the cell cycle regulator p27 but not p21 (Figure 6A).

To understand if the changes in the protein levels were caused by transcriptional regulation, RNA was purified from these cells and reverse transcribed into cDNA. A semi-quantitative PCR analysis was carried out using this cDNA. The results of the semi-quantitative PCR analysis show that the mRNA levels of Her-2, VEGF and cyclin D1 are indeed reduced, strongly indicating that the observed reduction in the levels of the respective proteins is due to transcriptional repression (Figure 6B).

<u>Responsiveness of chemo-resistant breast cancers to chemotherapy in presence of the KRAB recombinant proteins.</u>

We carried out WST-1 cell proliferation assays to characterize the effect of the cancer chemotherapeutic drug, doxorubicin, on SkBr-3 cells transfected with the KRAB-recombinant plasmids. Cells were cultured in 24 well plates and transfected with the plasmids in triplicates.

After 48 h, 100 nM doxorubicin was added to the cells and the incubation was continued for another 24 h to complete a total incubation period of 72 h. The procedure is described in the legends section. The data shows that doxorubicin induces an additive decrease in proliferation of the cells in presence of all constructs. This suggests that rather than a strong synergistic effect, the combination therapy of drug and KRAB-recombinant protein show a weak synergy. However, the additive effect may be highly beneficial for cancer therapy since the KRAB-proteins themselves show extremely potent inhibition of cancer cell proliferation. Note that the bar graphs represent proliferation changes in all the cells, while only a fraction of the cells (~20%) are transfected by the plasmid construct. In its present context, the data would be good representation of in vivo combination therapy because present day gene delivery systems would also deliver a therapeutic gene to only a fraction of the cells. However, it is desirable to develop better gene delivery systems for cancer therapy.

Key research Accomplishments:

Research accomplishments during the first year of report:

1. I used a high throughput screen (REPSA) to identify DNA sequences that bind to hairpin polyamides with highest affinity and specificity.
2. I also identified a hairpin polyamide (HPA-1) that shows strong interaction with the TATA box of the Her-2 promoter. Through an EMSA experiment, I showed that HPA-1 could displace the primary transcription apparatus (TBP in the TFIID complex) from binding to the Her-2 core promoter.
3. Luciferase reporter assay using a Her-2 promoter construct showed that HPA-1 indeed down-regulates Her-2 promoter activity, albeit at low levels. Co-transfection of the cells with the recombinant KAP2 repressor protein showed a higher fold down-regulation of the Her-2 promoter. This suggested that the AP-2 binding site is an important target for down-regulating Her-2/neu expression and also that the KAP2 repressor may have potential as a gene therapeutic.

Research accomplishments during the second year of report:

1. Through luciferase-reporter assays, I showed that HPA-1 and the KRAB-recombinant proteins could inhibit the transcriptional activity of the Her-2 promoter. Immuno-

fluorescence studies showed that the endogenous Her-2 expression was dramatically repressed by the KRAB-repressor proteins.

2. The hairpin polyamide HPA-1 by itself was not very effective in inhibiting the proliferation of Her-2 over-expressing breast cancer cells. The KRAB repressor proteins however were more effective in inhibiting the proliferation of the Her-2 over-expressing cells, but not Her-2 low expressing cell lines. Combination of both HPA-1 and the repressor proteins had a strong effect on the proliferation inhibition.
3. The KRAB-recombinant proteins strongly inhibited colony formation in Her-2 over-expressing SKBR-3 cells. In a TUNEL assay, the KAP2 and KAP2r transfected SkBr-3 cells showed apoptotic death. These results strongly suggest that proliferation inhibition by the KRAB repressor proteins could probably be due to the induction of programmed cell death. In the next year of report, detailed apoptosis assays would be carried out in the cells treated with the KRAB constructs and HPA-1 to determine how these agents cause apoptosis. This knowledge could provide impetus for their therapeutic development.

Research accomplishments during the current year of report:

1. Using FACS analysis, I was able to analyze proliferation inhibition by the KRAB-recombinants in a homogeneous population of the transfected cells. The high percentage of cells in sub-G1 phase of the cell cycle indicates that proliferation inhibition by KAP2 and KAP2r is due to apoptosis induction. This conclusion was substantiated through a caspase 3/7 detection assay, in which increase in caspase activity was monitored in presence of these constructs.
2. I carried out a colony formation assay in MCF-7 cells, similar to that reported earlier in SkBr-3 cells(figure 4, 2003 annual report). The data indicates that the KRAB-recombinants do not induce a sharp reduction in colony formation in the low Her-2 expressing MCF-7 cells. But the cells still seem to possess a growth disadvantage, indicating that apart from Her-2, other programs of gene expression relevant to cancer cell survival may be adversely affected by the KRAB-recombinants.
3. I developed a rapid FACS based transfectant screening protocol for isolating a homogenous population of transfected cells that express the KRAB-recombinants. Using these cells, I carried out western and semi-quantitative RT-PCR analysis for identification

of changes in the expression levels of Her-2 and other cell survival and cell cycle regulatory proteins. The goal was to identify other genes and programs of gene expression that are repressed by the KRAB-recombinants. I have successfully identified that the expression of VEGF and cyclin D1, along with Her-2 are affected by the KRAB-recombinants. This strongly suggests that other survival genes are also repressed by the KAP2 and KAP2r.

4. Treatment of Her-2 over-expressing breast cancer cells with a combination of KRAB-AP2 constructs and doxorubicin did not induce a synergistic effect (where the combined effect of two different therapeutics would have a geometric rather than an additive effect). This however may be an expected result since most of the transfected cells would anyway apoptose. Interpretation of this result as synergistic or additive is complicated by the fact that only a few cells are transfected by the plasmids, while doxorubicin essentially enters all cells.

Publications

Y.N. Vashisht Gopal and Michael W. Van Dyke

"A Combinatorial Determination of Sequence Specificity for Nanomolar DNA-binding Hairpin Polyamides".

Biochemistry, 2003 Jun 10;42(22):6891-903.

Reportable outcomes

Publications:

Y. N. Vashisht Gopal and Michael W. Van Dyke

"Repression of AP-2 dependent transcriptional programs induces apoptosis in breast cancer cells."

Manuscript under preparation for communication to ***EMBO Journal***.

Research projects and Grants:

This work was directly responsible for the development of several research projects in our laboratory, some of which are funded.

Conclusions

The hairpin polyamide HPA-1 is a DNA minor-groove binder with high affinity of interaction with the Her-2 core promoter. This molecule shows potent inhibition of TBP interaction with the core promoter. However, its in vivo activity was not as pronounced and it lacked therapeutic potential. The Her-2 promoter contains two AP-2 binding sites and the KRAB-AP2 recombinant proteins possessed exceptional affinity of interaction with the Her-2 promoter, due to their inherent helix-span-helix DNA binding domain of AP-2. The KAP2 and KAP2r proteins show potent inhibition of Her-2 promoter activity as was judged from Her-2 promoter reporter studies. Western and semi-quantitative RT-PCR analysis revealed that these proteins indeed show potent inhibition of Her-2 transcription. They also repress the transcription of other survival genes like cyclin D1 and VEGF.

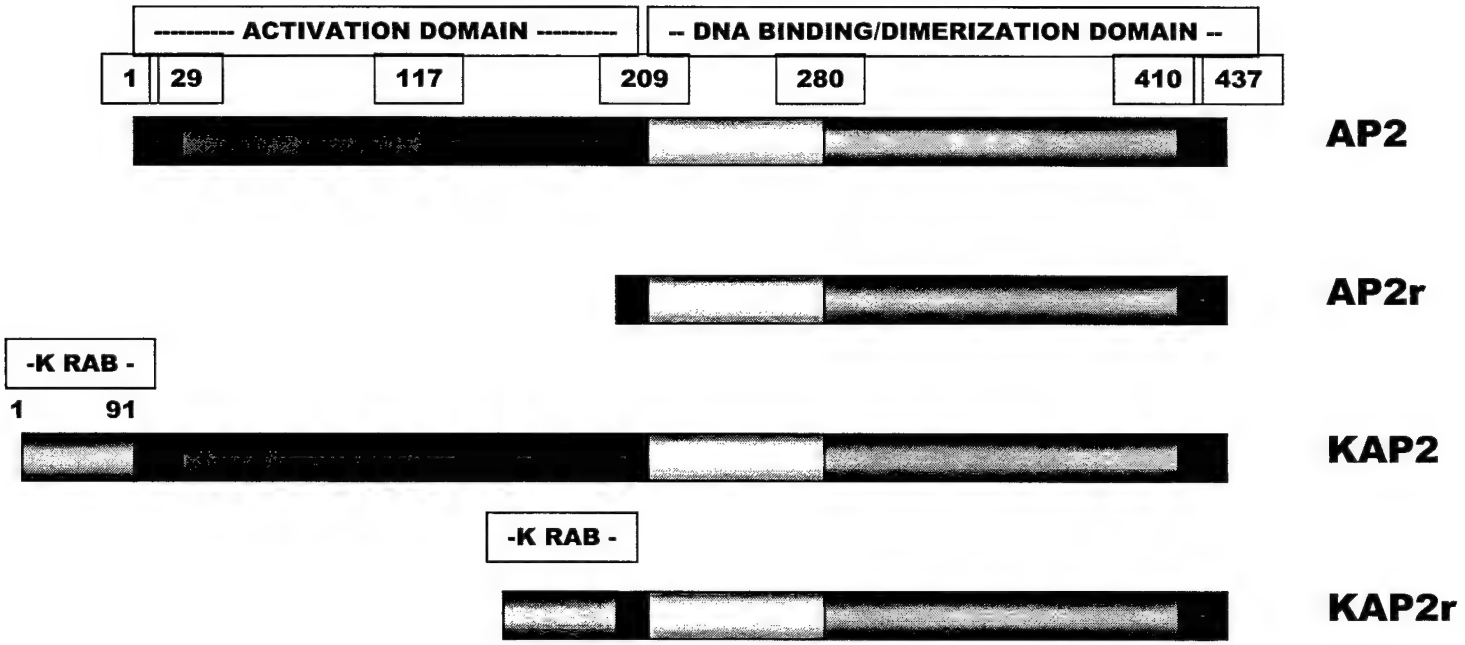
Unlike AP-2, which seems to induce a growth advantage to Her-2 overexpressing cancer cells, the KRAB-AP2 recombinants seem to induce a growth disadvantage. This is expected since they repress the expression of several survival proteins necessary for cancer cell growth and proliferation. Proliferation studies using WST-1 assays, FACS-cell cycle profile experiments and caspase assays showed that the KRAB-recombinants inhibit cell proliferation and induce caspase3/7 mediated apoptosis.

In summary, the KRAB-AP2 recombinant proteins possess the potential to be used as gene therapeutics for Her-2 over-expressing breast cancers. We are currently planning to expand the research for animal studies to determine their *in vivo* efficacy.

References

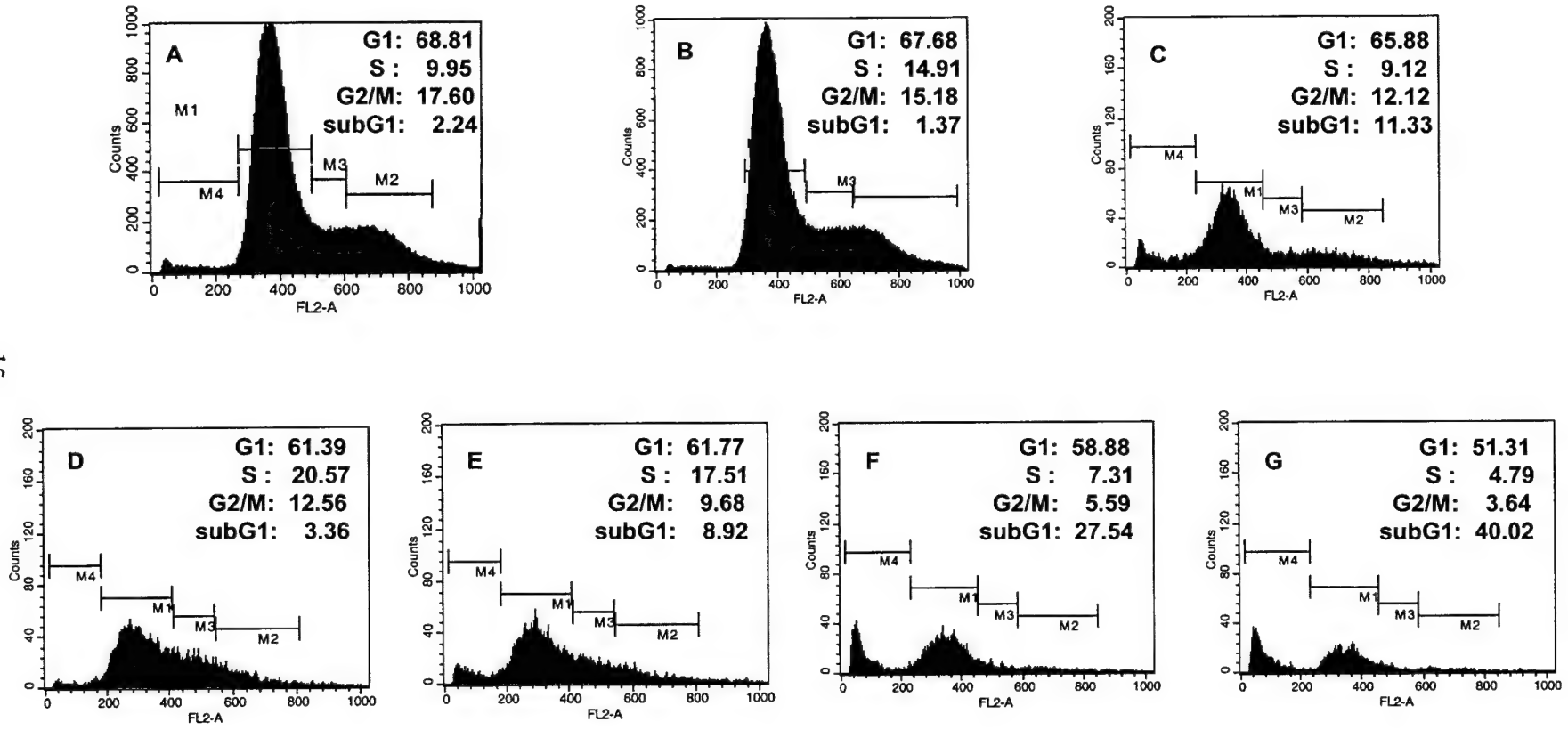
1. Yu, D. and Hung, M.C., Oncogene, 19:6115-6121 (2000)
2. Downward J, Yarden Y, Mayes E, et al., Nature 307:521-527 (1984)
3. Yarden, Y. Sliwkowski, M.X., Nat. Rev. Mol. Cel. Biol. (2001)
4. Olayioye MA, Neve RM, Lane HA, et al., EMBO J., 19:3159-3167 (2000)
5. Zhou, B.P. and Hung, M.C., Semin. Oncol., 30:38-48 (2003)
6. Hou L, Shi D, Tu SM, et al., Cancer Lett., 65:215-220 (1992)
7. Schneider PM, Hung MC, Chiocca SM, et al., Cancer Res. 49:4968-4971 (1989)
8. Weiner DB, Nordberg J, Robinson R, et al., Cancer Res., 50:421-425 (1990)
9. Yokota J, Yamamoto T, Miyajima N, et al., Oncogene 2:283-287 (1988)
10. Slamon DJ, Clark GM, Wong SG, et al., Science 235:177-182 (1987)
11. Slamon DJ, Godolphin W, Jones LA, et al., Science 244:707-712 (1989)
12. Ross JS, Fletcher JA, Bloom K.J. et al., Mol Cell Proteomics, 3:379-98 (2004)

Figure 1



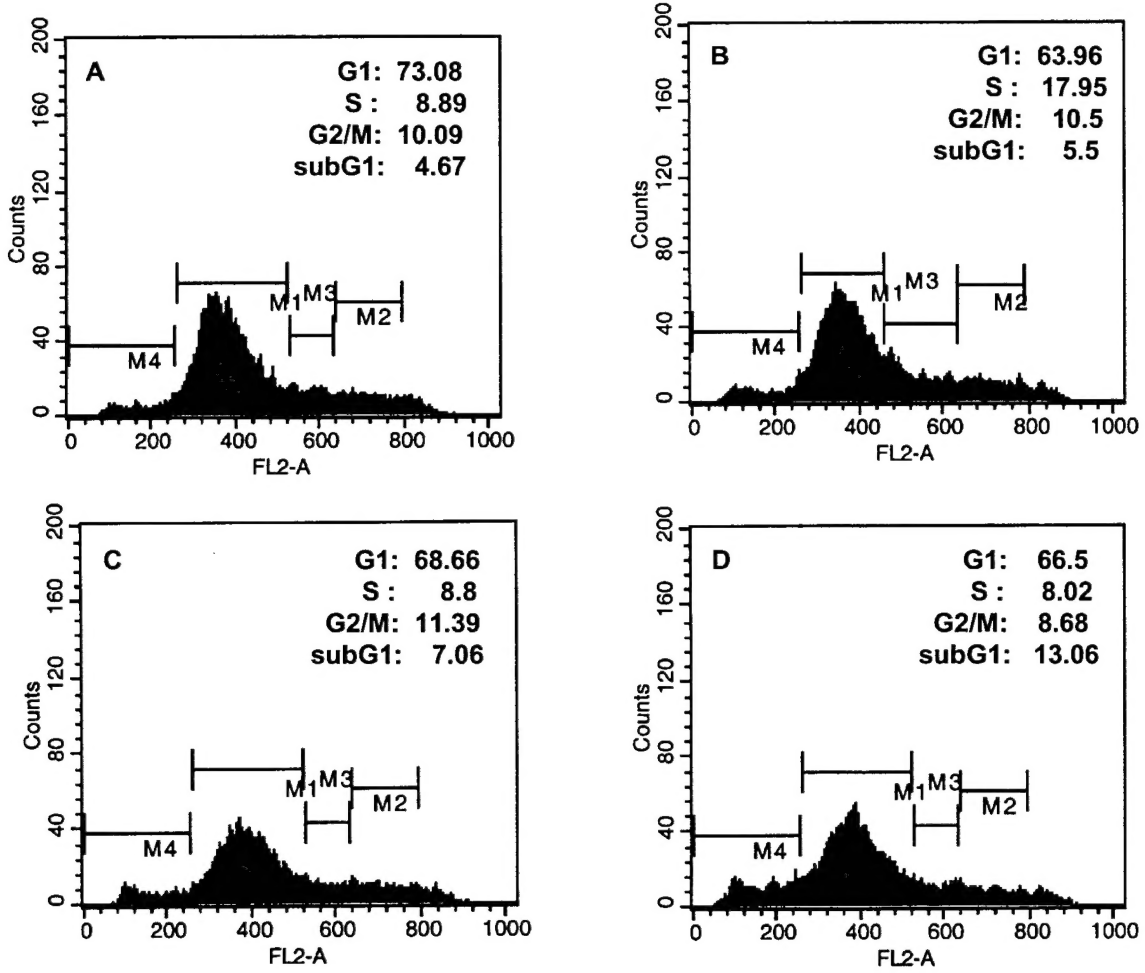
Shown above are the domain structures of the different AP-2 recombinant constructs cloned into a pCMV-tag vector. **AP2** is the human AP2alpha protein is a 437 amino acid, 52 Kda protein with a distinct activation domain and DNA binding/dimerization domain. The domain locations are indicated by the number of amino acids spanning each domain. The construct **AP2r** lacks the activation domain, but retains the DNA binding and dimerization properties. **KAP2** is the full length AP2alpha with a KRAB domain fused to the N-terminus of the protein. **KAP2r** is AP2r with a KRAB domain fused to the DNA binding/dimerization domain. Not shown above is another construct that expresses only the KRAB domain (named as **K**). Our studies have consistently shown that when **K** is expressed in cells, it localizes in the cytoplasm and has no apparent effect on cell physiology. Hence the **K** plasmid served as a good negative control for our experiments. An immunogenic FLAG-tag on the plasmids helps in easy immunogenic recognition of these proteins. The vector contains a G418 resistance marker to carry out colony formation assays and stable clone selection in G-418 antibiotic containing growth medium.

Figure 2



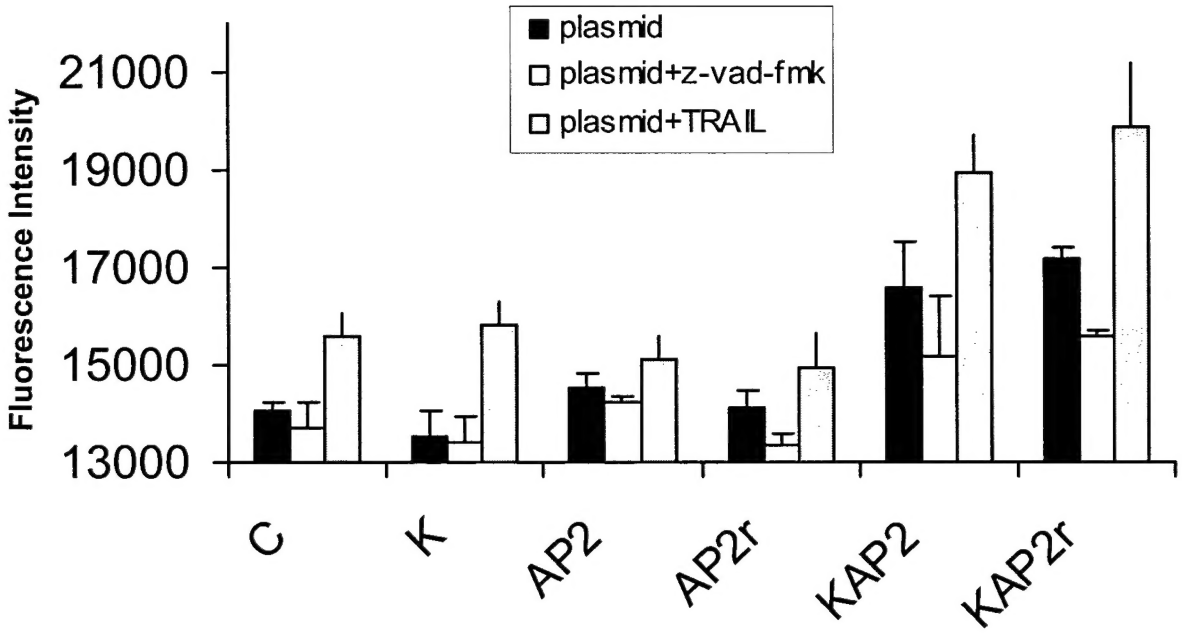
Transiently transfected SkBr-3 cells were incubated for 48 h, fixed and stained with FITC-anti Flag antibody for two hours. The cells were then stained with propidium iodide and a cell cycle profie was generated using flow cytometry. Figure A shows the profile of untransfected and transfected cells from AP2r transfected cells. Figure B shows the profile of untransfected cells alone. Figure C shows the profile of the AP2r transfected cells alone. Figure D, E, F and G are profiles of cells transfected with K, AP-2, KAP2 and KAP2r respectively.

Figure 3



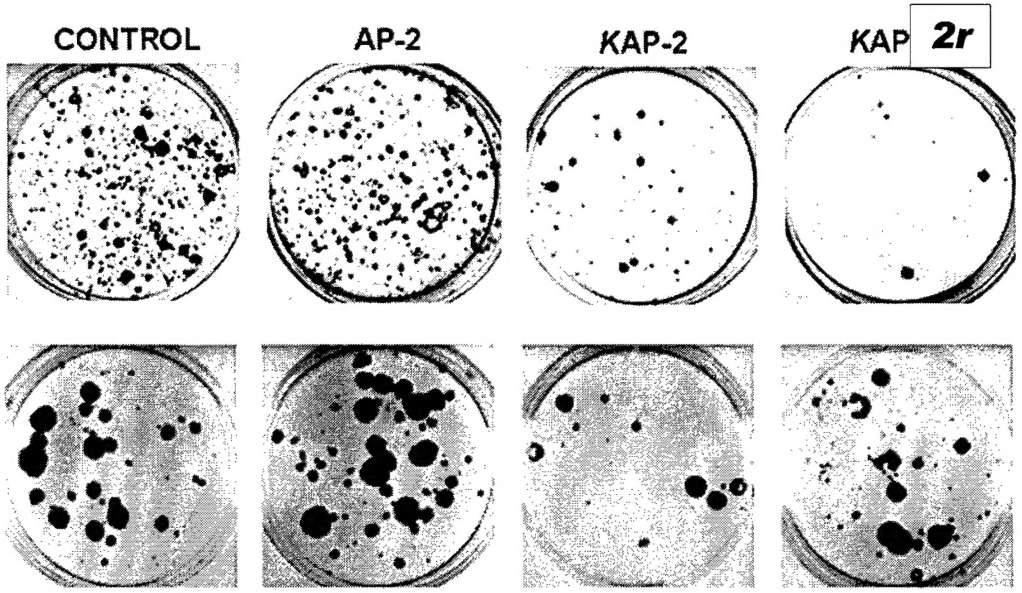
Cell cycle profile of K (**A**), AP2 (**B**), KAP2 (**C**) and KAP2r (**D**) transfected MCF-7 cells show lower numbers of cells in the sub-G1 phase of cell cycle compared to SkBr-3 cells. This suggests that the KRAB-recombinants readily induce apoptosis in high Her-2 expressing cells, while inducing lower levels of apoptosis in the low Her-2 MCF-7 cells.

Figure 4



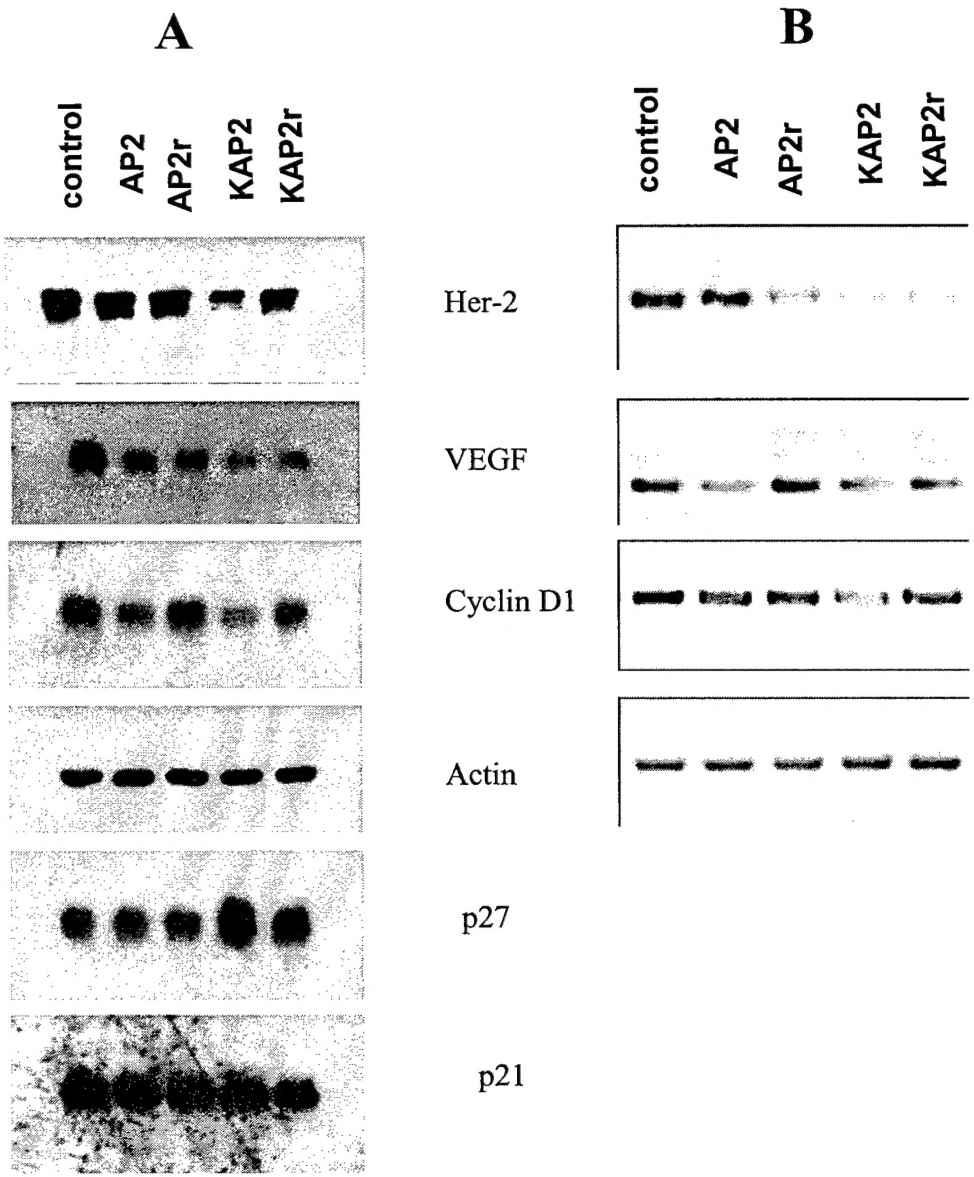
SkBr-3 cells were transfected with the plasmids as shown in the X-axis and incubated for 40 h. z-VAD-FMK (10μM) and TRAIL (50ng/ml) was added to the cells and incubated for 8 h (total incubation of 48 h). The cells were lysed and analyzed for caspase 3/7 activity. The data shows that KAP2 and KAP2r increase the caspase 3/7 activity indicating that they induce apoptosis.

Figure 5



Colony formation assay on SkBr-3 and MCF-7 cells shows that while AP-2 provides a growth advantage to the cells, KAP2 and KAP2r seem to provide a growth disadvantage. The SkBr-3 cells are more sensitive to the expression of KAP2 and KAP2r than the MCF-7 cells.

Figure 6



Western analysis (**A**) of the FACS sorted SkBr-3 cells shows that KAP2 and KAP2r reduce the protein levels of Her-2, VEGF and Cyclin D1, while increasing the levels of p27.
To verify if this effect was due to transcriptional repression, a semi-quantitative RT- PCR.analysis was carried out (**B**). The data shows that the cells expressing KAP2 and KAP2r indeed have lower levels of transcribed mRNA for Her-2, VEGF and cyclin D1. Currently, RT-PCR studies are being carried out to analyze the effect on p27, p21 and other proteins involved in cancer cell survival and apoptosis.